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(54) Title: DETECTION AND IDENTIFICATION OF DNA AND/OR RNA IN PROTEIN-CONTAINING SAMPLES

(57) Abstract

A method for isolating and concentrating DNA and/or RNA from samples containing large amounts of protein contaminants is disclosed. In a three-step process, sufficient DNA and/or RNA can be isolated from high amounts of protein to permit detection of nucleic acid at femtogram levels. A preferred detection method using peroxidase as label and TMB as substrate is disclosed.

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DETECTION AND IDENTIFICATION OF DNA AND/OR RNA IN PROTEIN-CONTAINING SAMPLES

Field of the Invention

The invention relates to assay procedures for the detection and identification of native and recombinant DNA or RNA in biological or production samples. In particular, it relates to detection of extremely small quantities of DNA or RNA in samples in which the DNA or RNA is overwhelmed by protein.

Background Art

The production of biological products from either recombinant or native sources often requires that the finished product contain little or none of the DNA and RNA associated with the cell producing it. This is particularly important if the protein is produced by a viral system which may contain DNA or RNA which is potentially infectious, or when the protein is produced by a recombinant immortalized cell, when the DNA of the transformed cell, in particular, may cause carcinogenic effects on subjects administered the product containing this impurity. Therefore, the ability to detect extremely small amounts of DNA or RNA in the presence of large amounts of protein is highly significant in these contexts.

Another set of instances in which such detection is important is in assessing and identifying a particular DNA or RNA, a DNA associated with disease, for example, in biological samples which intrinsically contain large amounts of protein. U.S. Patent 4,358,535 to Falkow discloses one approach to such detection. Indeed, the literature is extensive with respect to assays for particular DNAs. See, for example, Burke, R.D. et al., J Obstet Gynocol (1986) 154:982-989; Chen, J.Y. et al., Dr J Exp Pathol (1986) 67:279-288; Hypi'a, T. et al., Clin Lab Med (1985) 5:591-601; Yehiely, F.C. et al., Int J Cancer (1986) 38:395-403; McKeating, et al., J Virol Method (1985) 11:207-216; Anderson, M.J. et al., J Med Virol (1985) 15:163-172; Pollice, M. et al., Clin Lab Med (1985) 5:463-473; Tompkins, L.S. et al., Diag Microbiol Infect Dis (1986) 4:715-785; Kuritza, A.P. et al., J Clin Microbiol (1986) 23:343-349; Razin, S. et al., In Vitro (1984) 20:404-408; McLaughlin, G.L. et al., Am J Trop Med Hyg (1985) 34:837-840; and Ferreira, A. et al., Mol Biochem Parasitol (1986) 16:103109. The foregoing assays are of moderate sensitivity, and in general require use of radioisotopes in order to obtain a signal sufficiently strong to detect DNA at the picogram or femtogram level. One reason for this lack of sensitivity resides in the inability to obtain the DNA or RNA from the sample free of obscuring impurities. The present invention remedies this defect.

Disclosure of the Invention

The invention provides a method to isolate DNA or RNA free of obscuring impurities from samples wherein the DNA or RNA is in extremely small quantity in comparison to large amounts of protein present. In effect, the method of the invention provides an extremely simple

and effective DNA or RNA purification procedure which succeeds in retrieving nearly the entire amount of DNA or RNA present in an assay sample, despite the presence of high protein levels. The assay has been applied to detection of DNA at the femtogram level without the use of radioisotopes. By suitable labeling procedures, both DNA and RNA can be detected, and distinguished. Thus the ability to prepare materials in the substantial absence of DNA and RNA and to confirm this absence is assured. The availability of this assay, for example, might have prevented the incidence of latent viral infection in subjects administered human growth hormone which was later shown to have carried DNA from a highly pathological virus.

Accordingly, in one aspect, the invention is directed to a method to detect DNA or RNA in a sample containing large amounts of protein, wherein the method comprises the steps of digestion of the protein component, isolation of the DNA and/or RNA using chromatographic adsorption and elution, followed by further concentration of the DNA and/or RNA on a nylon support. The DNA and/or RNA thus covalently bound to the nylon support is then detected by any convenient means either while remaining on the support, or when dissolved away from it. The choice of nucleic acid modification determines whether DNA or RNA or both, or a specific subset of either, is detected.

Modes of Carrying Out the Invention

The essential steps involved in the method of the invention represent a combination of three processes, each known in the art as applied to other purposes, and uniquely combined according to the method of the invention to effect the efficient isolation and

concentration of DNA and/or RNA in the sample despite the presence of large amounts of protein.

The first step in the procedure is the digestion of the protein, thus permitting the DNA and/or RNA to be removed by chromatographic techniques in the second step. While protein digestion is, of course, known in general, its application to the isolation of DNA and/or RNA in the herein described protocol is new. In general, any efficient digestion procedure may be used, but it is particularly effective to employ a nuclease-free preparation of exoprotease, such as proteinase K or pronase, especially after treatment of the sample to expose the protein sequence, thus making the proteins more accessible to protease digestion. Suitable methods for such reduction involve the use of a chaotropic agent in the presence of a reducing agent. The use of such reagents should be chosen such that subsequent chromatographic separations are not affected. Likewise, salt conditions need to be such that they do not interfere with subsequent chromatographic and labeling procedures. We have found that most proteins can be digested in their native state using one or a combination of the above-mentioned proteases at a protease per sample protein ratio of 1:1000 for 1-8 hours at 55°C. Conditions will be optimized as understood in the art for individual protein preparations. For example, digestion of a preparation of a protein containing large numbers of disulfide linkages may benefit by treatment such as reduction and carboxymethylation prior to digestion. In some cases, we have found initial digestion with endoproteases such as trypsin and/or endoproteinase Glu-C, followed by the above-mentioned exoprotease digestion, to be efficient.

If the sample is thought to contain whole cells which harbor the DNA or RNA to be detected, the protease digestion step should also be preceded by, or include concomitantly, reagents and procedures designed to disrupt the cells. For example, lysozyme and a chaotropic agent, such as SDS, might be added to the protease digestion mixture, or the mixture may be sonicated or put through freeze-thaw cycles. In general, this additional set of reagents and conditions is needed when it is believed that the sample contains, for example, infectious organisms as whole organisms, or when a nonsecreted cellular product is the product to be assayed. As mentioned above, care must be taken in choosing such reagents to avoid interference with subsequent steps, thus reducing assay sensitivity.

In a typical embodiment, this initial step involves treating the sample with an effective amount of chaotropic agent, such as SDS, urea, or guanidine, in the presence of a reducing agent such as β-mercaptoethanol or dithiothreitol at high temperature, for example, by boiling, for 5-25 minutes. Urea or guanidine is preferred. If whole cell disruption is included, the additional reagents, sonication, or freeze-thaw are also used. The treatment time will vary with the difficulty involved in disrupting the cells, but 1-2 hours is an appropriate time for treatment with lysozyme; a shorter time for sonication. Cell disruption need not be completed before treatment with protease is started. The treated sample is then brought to a temperature compatible with an exoprotease and incubated with the protease at a ratio of about 1:1,000 for 18-30 hours at about 37°C, or other temperature appropriate for the protease. If desired, the digestion can be monitored using SDS-PAGE; pre-cast mini-gels (4-25%) are convenient for

this purpose. When the sample is satisfactorily digested, it is subjected to the second step in the method.

The second step involves nucleic acid concentration/purification by adsorption of the RNA and/or DNA onto a chromatographic support, followed by elution under appropriate conditions. Suitable supports are characterized by their ability to adsorb, efficiently, RNA or DNA or both. Practitioners of the art are familiar with a wide range of such supports, which include ion-exchange resins, such as DEAE-cellulose, reverse-phase columns, and general purpose adsorbents, such as hydroxyapatite. A large number of suitable supports are available. One particular support, NENSORB 20, is especially preferred. Methods for adsorption and elution of RNA or DNA to various supports are known in the art, and need not be repeated here. Yield and, therefore, overall assay sensitivity may vary with differing chromatographic chemistries; if quantitative determination is desired, calibration using spiked samples may be desirable.

The third step in the procedure involves concentrating the DNA and/or RNA by covalent linkage onto a blotting medium, in particular to a nylon blotting medium as opposed to, for example, nitrocellulose. Such blotting media may be derivatized and are commercially available under such trade names as Biotrace or Nytran. The use of nylon blotting media permits capture of a wide range of molecular weight RNA or DNA and is helpful in increasing the sensitivity of the assay. The freshness of the nylon membrane is particularly important. Loss of signal, presumably due to decreased nucleic binding capability, has been noticed with old membrane. It is preferred to purchase small amounts of membrane

material and to discard any remainder immediately upon signal degeneration. In placing the eluted RNA or DNA sample on the blotting medium, procedures are employed appropriate to the particular support used. See, for example, EP Application 221,308, which describes alkylation of nylon amide groups to aid in nucleic acid immobilization. Comparison of commercially available supports is found in the report of Khandjian, E.W., Biotechnology (1987) 5:165-167.

The foregoing steps constitute the essentials of the isolation and concentration procedure. Detection may be performed by attaching a label to the isolated DNA. The choice of method of attaching label will determine whether DNA or RNA or both are detected.

It should further be noted that if RNA is to be measured, precautions must be taken throughout the procedure to prevent degradation. The nature of these precautions--e.g., autoclaved glassware, wearing of gloves, RNAse-free reagents, etc., is well known in the art.

As used herein, "label" includes, depending on the context, the detectable moiety itself, such as a fluorophore or a radioisotope, and/or a substance which is an intermediate in the ultimate attachment of the detectable moiety. For example, in the examples below, poly-T is conjugated to the analyte DNA and then treated with biotin-modified poly-A tail, which then reacts with avidin/detectable moiety conjugate. Both the poly-A/biotin and avidin/detectable moiety are considered "label" herein.

One method to detect the isolated DNA or RNA is the classical method of hybridization to a kinased or nick-translated probe. This method may be convenient if a specific DNA or RNA is to be assayed, although it may

be less sensitive. However, if total DNA or RNA, regardless of nature, is to be detected, it is preferable to use a generic detection technique.

One such technique is commercially available, and involves conjugation of sample DNA with a poly-dT tail and subsequent hybridization of the tailed DNA to a poly-dA-bound label. Poly-dA may be conjugated to additional labeled polynucleotide, or, as in the commercially available "Bio-Bridge" system, to biotin. Detection is then by binding of the biotin to a labeled avidin component. There are, of course, many variations on this general approach. For example, if RNA, rather than DNA is to be detected, poly-U is used in place of poly-dT. In lieu of using such a kit, one might choose to purchase individual components (i.e., terminal transferase, dT, etc.) to achieve greater cost effectiveness as well as a greater versatility in terms of labeling (i.e., use of different deoxynucleotides for custom labeling of DNA, RNA, probes, etc.).

Thus, for detection of polynucleotides in general, a convenient procedure involves attaching an oligonucleotide tail to DNA or RNA contained in the sample. This is conveniently done prior to the application of the chromatographic eluate to the nylon blot. In the alternative, the concentrated RNA or DNA may be eluted from the nylon blot and the tails applied to the eluate from the nylon support. Under these circumstances, the DNA or RNA will be detected in the eluted solution. However, if the blotted DNA or RNA already contains the oligonucleotide tails, it can be detected on the surface of the support using an analogous system.

To tail the DNA or RNA, the RNA or DNA is first digested with an appropriate amount of RNase or DNase to generate fragments of convenient size--for

example, about 300 bp with 3'-hydroxy termini--before addition of the tailing polynucleotide along with the appropriate terminal transferase. The reaction is stopped, for example, with EDTA, and the unused polynucleotides removed, for example, by gel filtration (G-50 spin columns) or by chromatography on an appropriate support such as NENSORB-20.

The tailed RNA or DNA is then reacted with "label", which is correspondingly tailed with an oligonucleotide complementary to that used to tail the sample RNA or DNA. For example, if poly-dT tails are used, as in the commercial Bio-Bridge system, poly-A tails are used for the label. However, alternate configurations are, of course, equally workable, so long as the two tails are complementary. For example, ATTATTATT polymers would homologously bind to TAATAATAA; for RNA, if it is messenger RNA, poly-A tails may already be included in the sample. Any conveniently synthesized polynucleotide, along with its complement, can be used as the pair.

The "label" may be a detectable moiety directly attached to the oligonucleotide, such as a fluorophore or isotope-labeled nucleic acid, or the attached label may be an intermediate specific-binding material which then forms the bridge to the ultimate detectable species. For example, in the commercially available system, this "label" is biotin, which is then responsible for attachment of the detectable moiety conjugated to avidin, which specifically binds to the biotin portion. Also usable would be, for example, an immunoglobulin specifically reactive with a detectable moiety, or other specifically binding intermediate.

If a specific type of DNA or RNA is to be detected, the generic method can only be applied after

taking account of the need for specific interaction with the particular DNA or RNA to be detected. Thus, for example, if the presence of a particular DNA is desired to be detected, the untailed DNA in the nylon blot support is detected by a labeled probe which hybridizes to the desired DNA. The probe may be labeled in a variety of ways, or may be an intermediate in the binding of label, as in the application of the BioBridge system. It is then, the probe that is oligonucleotide-tailed, e.g., dT-tailed, if the system is to be used for subsequent labeling using Bio-Bridge poly-dA tailed biotin, for example.

The test herein also describes a method by which the soluble peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) can be used for detecting immobilized conjugate in a blot system. TMB, as described below, can be used to increase overall sensitivity of the assay. In addition, using buffering conditions as stated below, the TMB/H_2O_2 system displays a far greater stability than conventionally used substrates such as aminoethylcarbazole, 4-chloro-1-naphthol, etc. TMB is less hazardous than other commonly used peroxidase substrates

If RNA is to be distinguished from DNA, RNA-specific label, such as poly-dU, may be used. The specificity of the labeling is determined in large part by the specificity of the enzyme used in securing the tail to the target analyte. Thus, if poly-U is used in conjunction with an RNA-specific terminal transferase, only the RNA present in the sample would be labeled. If it is desired to detect both DNA and RNA, label would need to be provided suitable for both forms of the oligonucleotide.

Examples

The following examples are intended to illustrate but not to limit the invention.

Example 1

Detection of DNA in the Presence of Bovine Serum Albumin

Various levels of human placental DNA in amounts between 100 fg-1 ng were spiked into 1, 2, 5, 10 and 25 mg/ml BSA solutions. One ml samples were boiled for 10 minutes in the presence of 5% B-mercaptoethanol and 1% SDS. The samples were brought to room temperature, were treated with proteinase K (Sigma Chemical Co., St. Louis, MO) at a ratio 1:1,000 in 1.5 ml microfuge tubes for 24 hours at 37°C. Digestion was shown to be complete by monitoring on 8-20% linear gradient SDS-PAGE.

The samples which had been digested with proteinase K were passed over NENSORB 20 cartridges obtained from DuPont according to the manufacturer's protocol. Briefly, the resin was prewetted with 2 ml HPLC grade methanol and equilibrated with buffer containing 100 mM Tris-HCl, 10 mM triethylamine, 1 mM dipotassium or disodium EDTA (pH 7.7). The sample was diluted in 4 parts of the same buffer and applied to the column. Following washing with 3 ml of the same buffer, the column was washed with 6 ml HPLC grade water to remove excess salt. The bound DNA was then eluted with 50% methanol and collected in a total of 400 µl or less. The samples were dried under vacuum to complete dryness.

Since total DNA was to be detected, the dried samples were labeled using poly-dT. The samples were reconstituted with 5 ml of 10 mM Tris-HCl, pH 7.5 containing 1 mM EDTA and labeled with the Bio-Bridge

labeling system (Enzo Biochem Inc.) as follows: the DNA was digested 5 minutes at 37°C with a 1:5,000 solution of DNase to generate fragments, averaging approximately 300 bp, with free 3' hydroxy termini. The enzyme was inactivated by incubating at 65° for 5 minutes and homopolymers of dTP were added to the 3' OH termini using DNA terminal transferase for one hour at 37°C. The reaction was stopped with EDTA, and unreacted polydT removed using gel filtration. (These samples are stable at -80°C or after drying down.)

The labeled DNA was then applied to the nylon blotting medium as follows: the labeled DNA was diluted 1:10 to final volume of 100 µl in 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA. Ten fold serial dilutions were made to obtain various levels of DNA for a standard curve. Ten µl of 3 M NaOH were added to each tube with subsequent incubation for 5 minutes at room temperature before addition of 55 µl of freshly prepared 3 M ammonium acetate, pH 7. The entire 165 µl was applied to Biotrace or Nytran using a dot blot apparatus, and the blot was washed twice with 1 M ammonium acetate, pH 7, and air dried on a nonabsorbent surface. The dried membrane was then baked for 1-2 hours at 80°C. (The treated membrane can be stored at -80°C for at least two weeks.)

For detection, the blots were wetted with 1 x SSC, and the Bio-Bridge labeling intermediate (poly-dA-biotin) was diluted 1:50 in 1 x SSC, 0.1% SDS and placed on the blot for 2 minutes at room temperature. After two minutes an equal volume of 1 x SSC, 0.1% SDS was added and incubated for an additional two minutes. The blots were then washed as follows: (all washes contain 0.1% SDS and were at 47-48°C) once with 0.5 x SSC and 5 times with 0.2 x SSC. The blot was washed twice

at room temperature for 3.5 minutes per wash in 0.2x, 1x and 2 x SSC, and then blocked for 30 minutes in 1 x PBS 2% BSA, 0.1% Triton X-100, 5 mM EDTA. The avidin horseradish peroxidase complex, DETEK1-hrp complex (ENZO) was diluted 1:250 with complex dilution buffer and incubated with the blot for 60 minutes at room temperature. The blot was then washed gently three times for five minutes per wash in high salt washing buffer (10 mM potassium phosphate pH 6.5, 0.5 M NaCl 0.05% Triton X-100, 1 mM EDTA, 0.1% BSA), transferred to a clean dish and washed twice with 2 x SSC, 0.1% BSA, 0.05% Triton X-100, 1 mM EDTA. The blot was incubated in freshly prepared reaction mixture (250 µl 1% H_2O_2, 200 µl 20 mg/ml aminoethylcarbazole in 10.0 ml 100 mM sodium acetate pH 4.5). The signal developed within ten to thirty minutes and was read using a reflectance mode densitometer.

Table 1 shows the ability of the assay to detect even femtogram amounts of DNA in the presence of as much as 25 mg/ml BSA (-CTL represents unspiked BSA; +CTL is BSA spiked with 1 pg λ-phage DNA).

Table 1
Recovery of DNA from Spiked BSA Solutions

Concentration of DNA Spike	mg/ml BSA				
	1mg/ml	2mg/ml	5mg/ml	10mg/ml	25mg/ml
1ng	++++	++++	++++	++++	++++
100pg	++++	++++	++++	++++	++++
10pg	++++	++++	+++	+++	+++
1pg	+++	+++	++	++	++
100fg	+++	++	++	++	++
-CTL	-	-	-	-	-
+CTL	+++	+++	+++	++	++

Example 2

Detection of Various DNA Types

The procedure of Example 1 was repeated using, in addition to human placental DNA, salmon sperm and lambda phage DNA all in the presence of 50 mg/ml BSA. As shown by the results in Table 2, femtogram amounts of all three DNA types in the presence of 50 mg/ml BSA is detectable.

Table 2

Recovery of Human Placental, Salmon Sperm, and Lambda DNA Spikes from 50 mg/ml BSA Solutions

DNA Type	1ng	100ng	10pg	1pg	100fg	10fg	1fg
Human placental	++++	++++	++++	+++	++	±	bkg
Salmon sperm	++++	++++	++++	+++	+++	+	bkg
Lambda	++++	++++	++++	+++	++	±	bkg

Example 3

Process Purification Monitoring

Two recombinant hybridoma cell lines are examined through product purification schemes for the presence of DNA. The DNA content in the preparation at various stages of chromatographic purification was monitored using the method described in Example 1. The results, shown in Table 3, reflect a steady diminution in DNA content progressive stages of purification.

Table 3

Evaluation of Process Purification Samples for DNA Content

Process Purification Stage	Starting Material	Concentrate	1st Col Flow Through	1st Elution	1st 1M Wash	2nd Col Flow Through	2nd Elution	2nd 1M Wash
Cell line 1	+++	++++	+++	++	++	+	ND*	+
Cell line 2	+++	++++	+++	++	++	++	+	+
-CTL	-							
+CTL	+							

*No DNA detected.

Example 4

Protein Digestion

Initial characterization of protein removal conditions is performed empirically as set forth below. It is obvious from these results that different proteins may, in fact, require quite different digestion conditions for their complete removal. Failure to efficiently remove protein may seriously compromise subsequent nucleic acid concentration and labeling steps.

Digestion of Proteins at 55°C

Bovine serum albumin (BSA) was prepared to 3.5 mg/ml in HPLC-grade water. Murine IgG was prepared to 5.5 mg/ml in HPLC-grade water. One hundred microliters of these samples were digested in separate tubes with 5 μg Proteinase K (set 1) or pronase (set 2) for various time points (0, 30, 60, 120, 300 and 400 minutes) at 55°C. Controls for each included protein stored at -80°C and protein with no protease incubated for 480 minutes at 55°C so that thermal denaturation or endogenous proteolytic activity could be monitored. At designated time points, samples were removed from the 55°C water bath and quick frozen in dry ice-acetone.

Digestion efficiencies were monitored using 3-27% linear gradient SDS-polyacrylamide gels (SEPRAGELS) obtained from Integrated Separation Systems (Hyde Park, MA). Gels were visualized by Coomassie Blue R-250 staining.

Digestion of murine IgG was shown to be complete at 30 minutes (proteinase K) and 60 minutes (pronase). Digestion of bovine serum albumin, however, required extended digestion times -- 300 minutes for proteinase K, whereas complete digestion was not achieved for pronase.

Pretreatment Protocols

In a second digest procedure, 100 µl of bovine serum albumin (BSA) at 5 mg/ml was first digested with 5 µg of trypsin, chymotrypsin or endoproteinase Glu-C (Protease V8) for 30 minutes at 37°C. Following this predigestion, 5 µg of proteinase K was added to each tube. The tubes were then incubated for 30 minutes at 55°C, and the digests were handled and analyzed as stated above.

Results from this study showed that a tryptic predigest, followed by digestion with proteinase K resulted in sufficient removal of protein from the sample in only one hour, an improvement over the digestion protocol described above. It should be noted that predigests with chymotrypsin or V8 were not sufficient alone; at least 50-60% of the starting total protein remained at the end of the pretreatment.

Example 5

Use of Reflectance Mode Densitometry to Assign Quantitative Values to Colorimetric DOT BLOTs

We have performed analysis of nonradiometric DNA DOT BLOTs using a Bio-Rad Densitometer in the reflectance mode and accompanying data-capture software to assign relative values to various samples. Briefly, human placental DNA was poly-T labeled, immobilized on nylon membranes, and detected using DETEK1-hrp as described above (Example 1). Resultant blots were air dried and densitometrically scanned in the reflectance mode. Data were integrated and reported as percent area. Data have been compiled from three dilution series of 10, 1 and 0.1 pg DNA. These were run on three different occasions using three different sets of

reagents and solid supports. Numerical assignments are given as % area obtained directly from the densitometric analysis report. Data are presented in Table 4.

Table 4
Densitometric Comparison of DNA DOT BLOTs Expressed as % Area

DNA Content (pg)	% Area Run 1	% Area Run 2	% Area Run 3
0.1	6.31	7.19	3.18
1.0	11.80	10.61	5.58
10.0	27.30	25.40	15.29

A second experiment was done in which control recovery was examined using densitometric assignment of % area. Two types of DNA were used in this study: salmon sperm and λ phage DNA. The salmon sperm DNA was diluted to 0.2, 2.0 and 100.0 pg whereas λ DNA was diluted to 0.1 and 1.0 pg. These were labeled and visualized as described above and scanned densitometrically. Recovery values are given in Table 5.

Table 5
Recovery of DNA Controls Using Densitometrically Assigned Values

Salmon Sperm DNA (pg)		λ DNA (pg)	
Expected	Obtained	Expected	Obtained
0.2	0.12	0.1	0.23
2.0	1.6	1.0	1.01
100.0	102.0		

Example 6

Comparison with Commercially Available DNA Assay

In order to better assess the reliability of this assay, we have compared several process

purification samples from our manufacturing facility using the assay described herein as compared with the commercially available Chemiprobe assay (FMC). Samples tested compared favorably and gave, essentially, identical values for each assay. Values are presented in Table 6.

Table 6
Comparison of DNA Levels in Process Purification Samples in the Invention Assay and the FMC Chemiprobe Assay

	DNA Content	
Sample	Invention Assay	FMC Chemiprobe Assay
1	8.9 pg/ml	7.5 pg/ml
2	8.7 pg/ml	5.0 pg/ml
3	3.1 pg/ml	2.0 pg/ml
4	4.0 pg/ml	3.5 pg/ml
5	4.2 pg/ml	4.0 pg/ml

Example 7

Use of 3,3',5,5'-Tetramethylbenzidine as the Preferred Peroxidase Substrate

We have found that the use of 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate system for peroxidase visualization of DNA on nylon solid supports to be superior to other peroxidase substrates such as aminoethylcarbazole as supplied in the ENZO Bio-Bridge A labeling kit. The protocol we use has aided in the assay sensitivity and is run as follows.

Following hybridization with the poly A-biotin probe (Example 1), the nylon membranes are blocked in an effective solution such as 0.3% Brij-35, 5% non-dairy creamer in 10 mM Tris-HCl pH 7.5 for one hour at 50°C. They are processed according to standard procedures as described in Example 1 by adding DETEK1-hrp complex (ENZO) diluted 1:250 with complex dilution buffer,

followed by incubation and washing. The blot is then developed using the visualization system prepared as follows:

#1 Substrate Buffer (stable 2 months at ambient temperature) is prepared by bringing 50 mM citric acid, 50 mM boric acid, to pH 4.0 with 3 N NaOH;

#2 Reaction Buffer (stable 1 month at ambient temperature) is prepared by adding 160 µl 30% H_2O_2 to 400 ml Substrate Buffer;

#3 Substrate is 4 mg/ml (w/v) TMB in 0.1 N HCl;

#4 Visualization Buffer is made immediately prior to use by mixing 400 µl TMB stock (#3) into 10 ml Reaction Buffer (#2).

Following the final membrane wash, the membrane is allowed to sit on Whatman #1 filter paper (or equivalent) for 2-5 minutes to remove excess moisture. The blot is reinserted into the DOT BLOT apparatus and wells filled with 200 µl visualization buffer. Once desired intensities are reached, development is stopped by applying vacuum to remove substrate. Blots are air dried prior to analysis. The signal obtained is stable for up to 5-6 hours. Analysis of results, therefore, should be completed within this time frame.

Control callibration curves were obtained using the TMB substrate for assay of human placental DNA and salmon sperm DNA standards. The assay was conducted as set forth in Example 1, except for the reaction mixture for visualization, as described above. The results showed linearity when plotted on semilog paper over four logs, and the results were reproducible, as set forth in Table 7.

Table 7

TMB Substrate System Assays

% Peak Area*

pg	Human Placenta DNA	%CV†	Salmon Spleen DNA	%CV†
1000	18.0 ± 0.97	5.4	16.9 ± 0.19	0.8
100	13.9 ± 1.43	10.3	15.4 ± 1.23	8.1
10	11.3 ± 1.53	13.6	10.9 ± 1.08	4.9
1	7.7 ± 0.72	9.0	9.1 ± 0.81	8.8
0.1	7.3 ± 0.42	5.8	6.9 ± 0.54	7.7

*These values represent the ratio of intensity of the designated peak to the total for all peaks in the surface scanned. Accordingly, the values are only meaningful relative to those for other peaks on the same scanned surface.

†%CV is the coefficient of variation over the individual determinations in the assay conducted n times; in this table, n=4.

CLAIMS

1. A method to isolate and concentrate DNA and/or RNA from samples containing protein which method comprises:

(a) digesting the sample with an exoprotease;

(b) applying the digested sample to a chromatographic adsorbent capable of adsorbing the DNA and/or RNA, and eluting the DNA and/or RNA therefrom; and

(c) covalently binding the eluted DNA and/or RNA to a nylon support.

2. The method of claim 1 which further includes detecting the DNA bound to the nylon support.

3. The method of claim 2 wherein the DNA and/or RNA is detected by conjugating said DNA and/or RNA to an oligonucleotide and treating with complementary oligonucleotide-tailed label.

4. The method of claim 3 wherein the label is peroxidase..

5. The method of claim 4 wherein the peroxidase is measured using TMB as substrate.

6. The method of claim 1 wherein the sample in a) is pretreated with additional proteolytic enzymes selected from trypsin, chymotrypsin and protease V8.

7. The method of claim 1 wherein the sample containing DNA and/or RNA is treated with a chaotropic agent and a reducing agent and with proteinase K until

completely digested; the digest is applied to a chromatographic support, and the DNA and/or RNA eluted, the eluted DNA and/or RNA conjugated to oligonucleotide tails, and the tailed DNA and/or RNA applied to nylon support.

8. The method of claim 1 wherein the DNA and/or RNA covalently bound to nylon support is detected by hybridization to a probe specific for a desired RNA and/or DNA.

9. A method to detect DNA-tailed peroxidase which comprises contacting said peroxidase with substrate TMB and H_2O_2 under conditions wherein the enzyme is catalytic.

10. A kit suitable for conducting the procedure of claim 1 which includes an exoprotease, a chromatographic absorbent capable of absorbing the DNA and/or RNA, and a nylon support, along with instructions as to the conduct of the procedure, and reagents for detecting the DNA and/or RNA.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/01130

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 1/00; C07H 15/12
US CL : 435/6; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	210/656,660; 435/6,18,23,28,259,270,803,810; 436/94,810; 536/27 935/2,6,19,20,77,78

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	R.L.P. ADAMS ET AL, "The Biochemistry of Nucleic Acids," published 1976, by Academic Press (New York), See pages 50-82, especially 50-52, 57-61, 65-67.	1-8,10
Y	R.F. SCHLEIF ET AL, "Practical Methods in Molecular Biology," published 1981, by Springer-Verlag (New York), See pages 15,16,89,90,93,99,100,108.	1-8,10
Y	T. MANIATIS ET AL, "Molecular Cloning A Laboratory Manual," published 1982, by Cold Spring Harbor Laboratory, (New York), See pages 188-196, 217-220, 280-281.	1-8,10
Y	US, A, 4,652,517 (SCHOLL) 24 March 1987, See Column 9, lines 20-42.	1-8,10

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report[9]
21 June 1988	21 JUL 1988
International Searching Authority[1]	**Signature of Authorized Officer**[10]
ISA/US	Margaret Moskowitz Margaret Moskowitz

Form PCT/ISA/210 (second sheet) (October 1981)